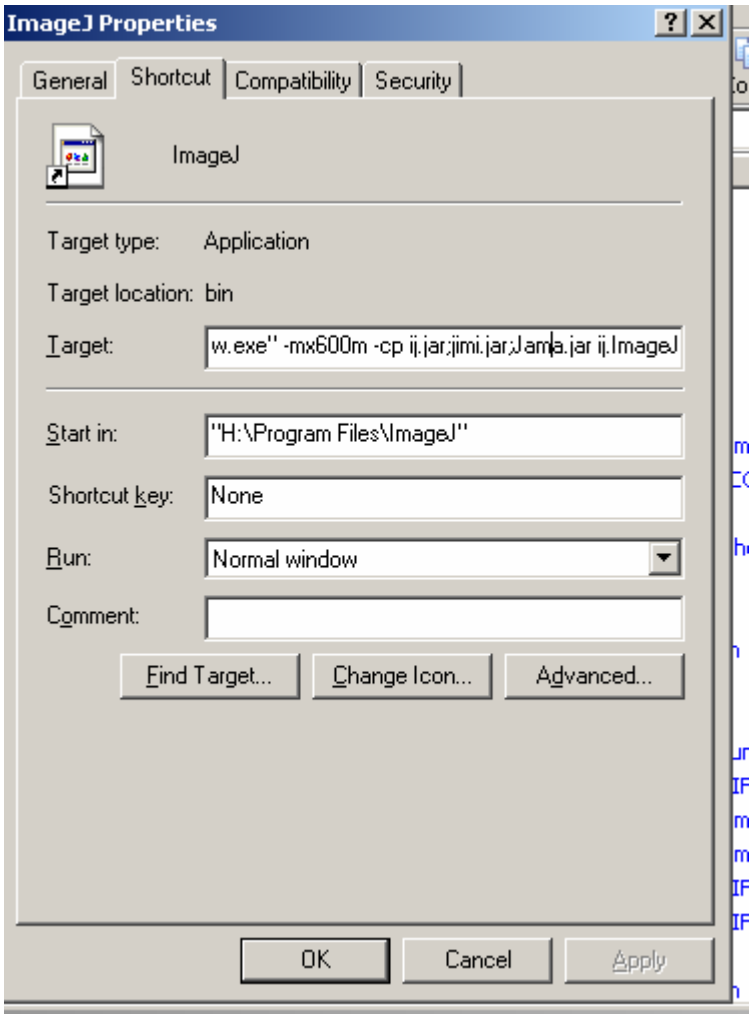
Instructions for Biolumunmixing v. 1.0

Installation:

1) Copy the file biolumunmixing_.java to the plug-in directory for ImageJ. This is usually as folder labeled Plug-In in the main ImageJ folder.
2) Go to the website http://math.nist.gov/javanumerics/jama/ and download the zip archive for the latest version. Unzip the file and install into your ImageJ folder. The program will not run without this file
3) Edit the shortcut that opens ImageJ by right clicking on the icon and choosing

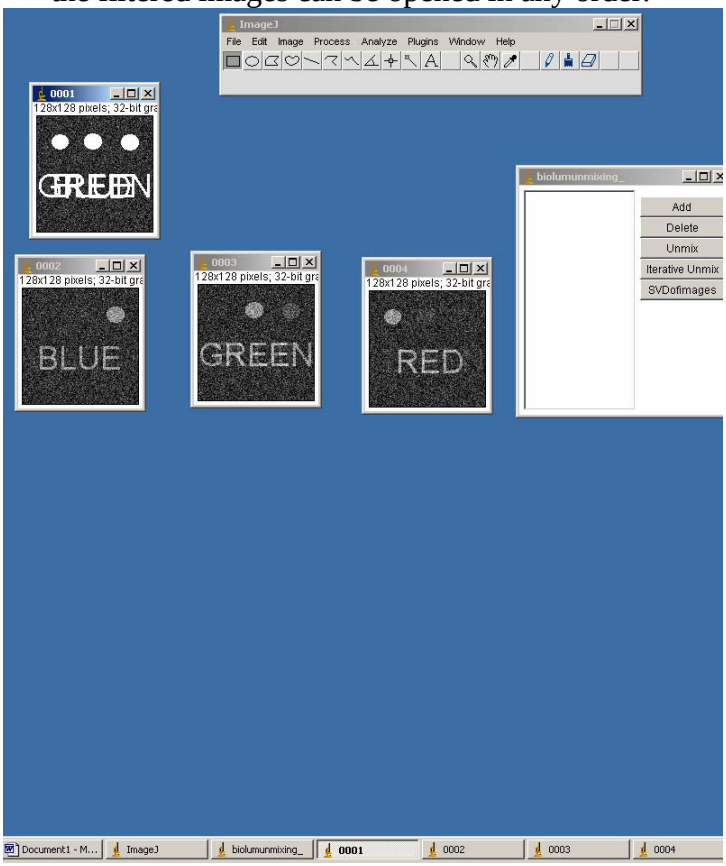


properties. Make sure that the Target line includes “Jama.jar”. Also, dedicate at least several hundred megabytes of memory if you will be deconvoluting 32 bit images. For example in the command line above there are 600 megabytes of memory allocated to ImageJ.

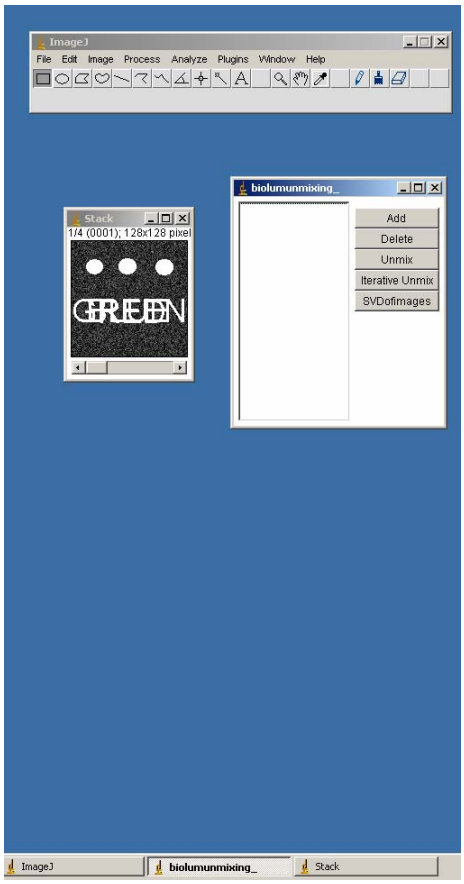
4) Open ImageJ; open the plugin’s folder and click “Compile and Run”; compile biolumunmixing_.java

Using the program:

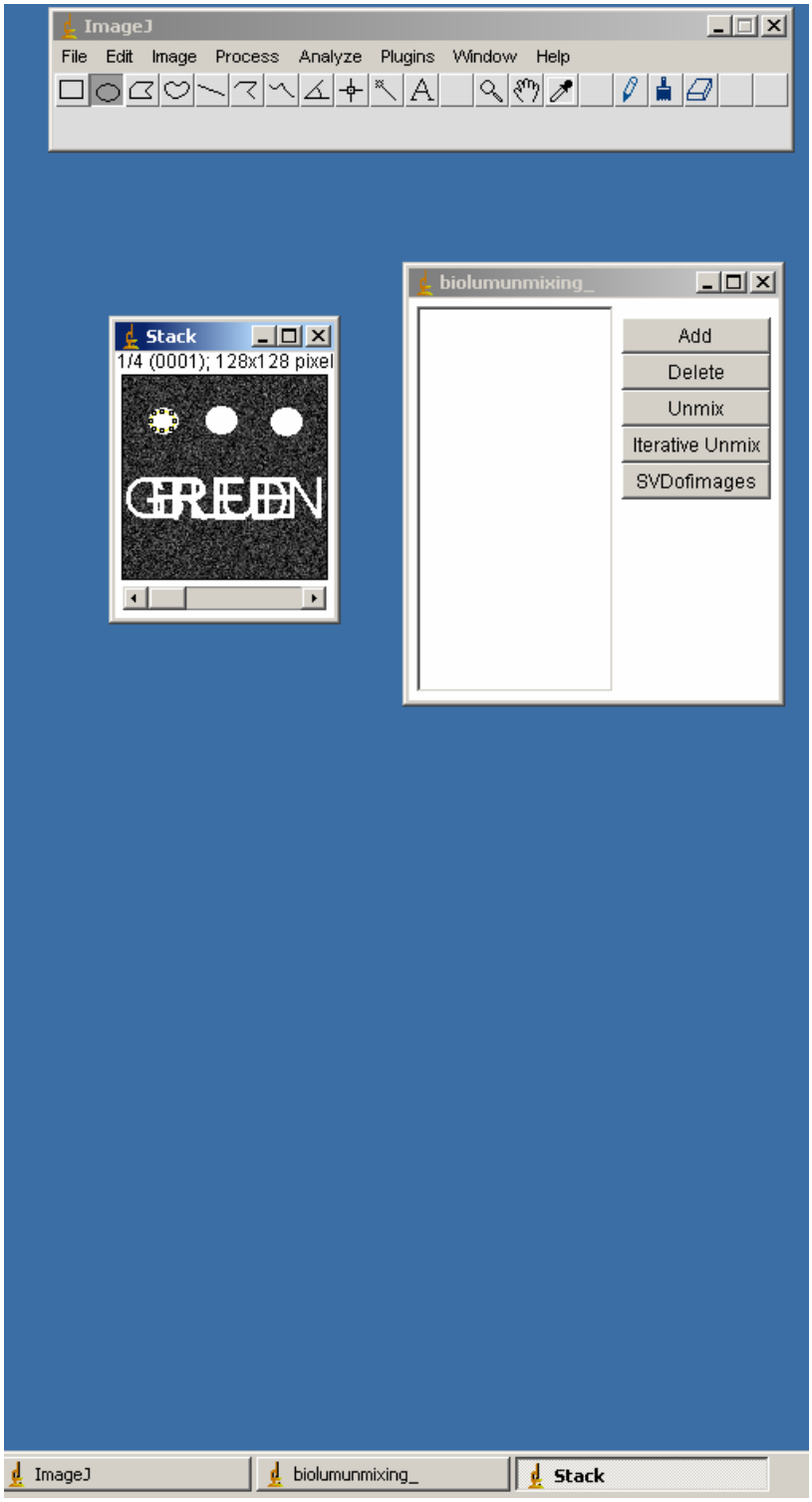
1) Open your bioluminescence images (preferably already corrected for lense warping). THE UNFILTERED IMAGE MUST BE OPENED FIRST. After that the filtered images can be opened in any order.



2) Open the Image Menu, then the Stack submenu then select convert images to stack.

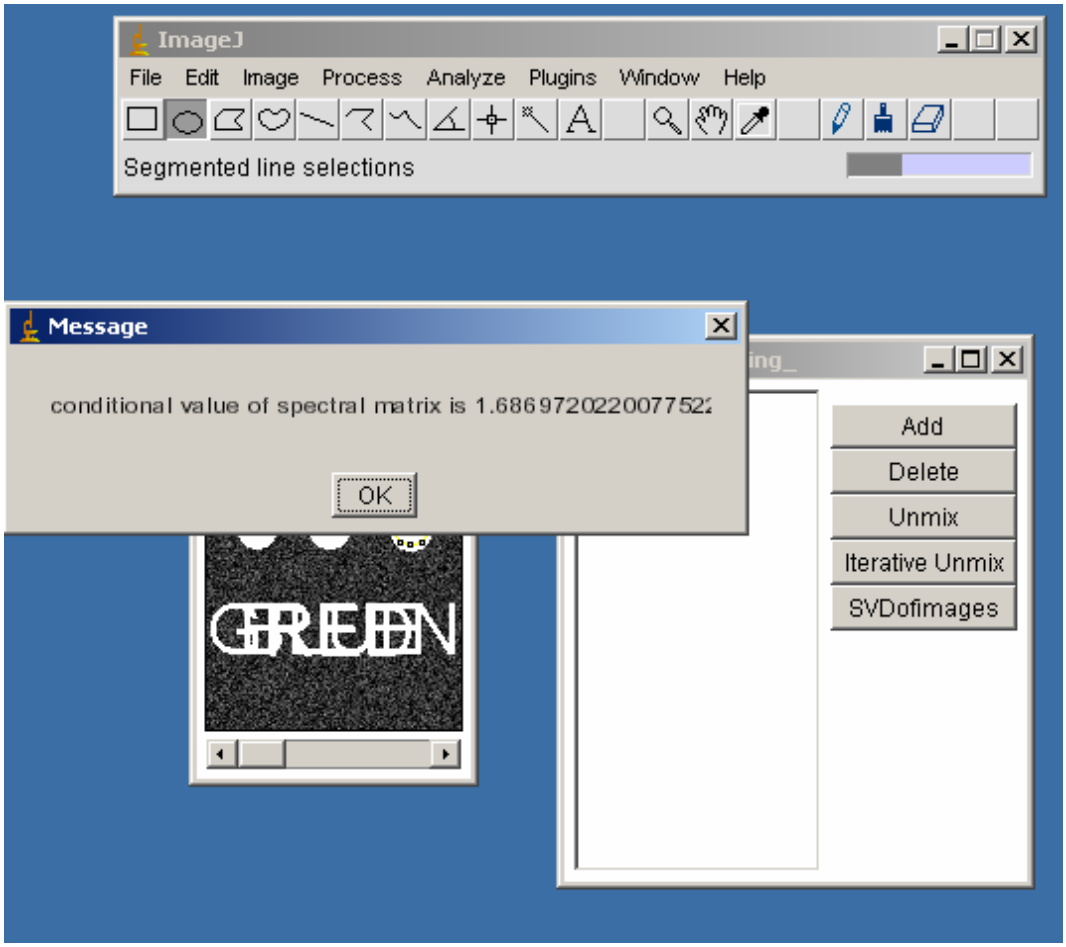


3) At this point you may process your images with any “filters” that you find useful. (ex. Gaussian blurring, fourier transform etc.) However, all of the images must be processed identically, so its best to the processing the whole stack at once.
4) If you are using a coelenterazine analogue in any of your wells, I recommend subtracting out the background from each image based upon the negative control well at this stage.
5) Draw a region of interest (ROI) using any of the ROI tools around a region that has only one type of luciferase. After the ROI has been draw click the Add button.

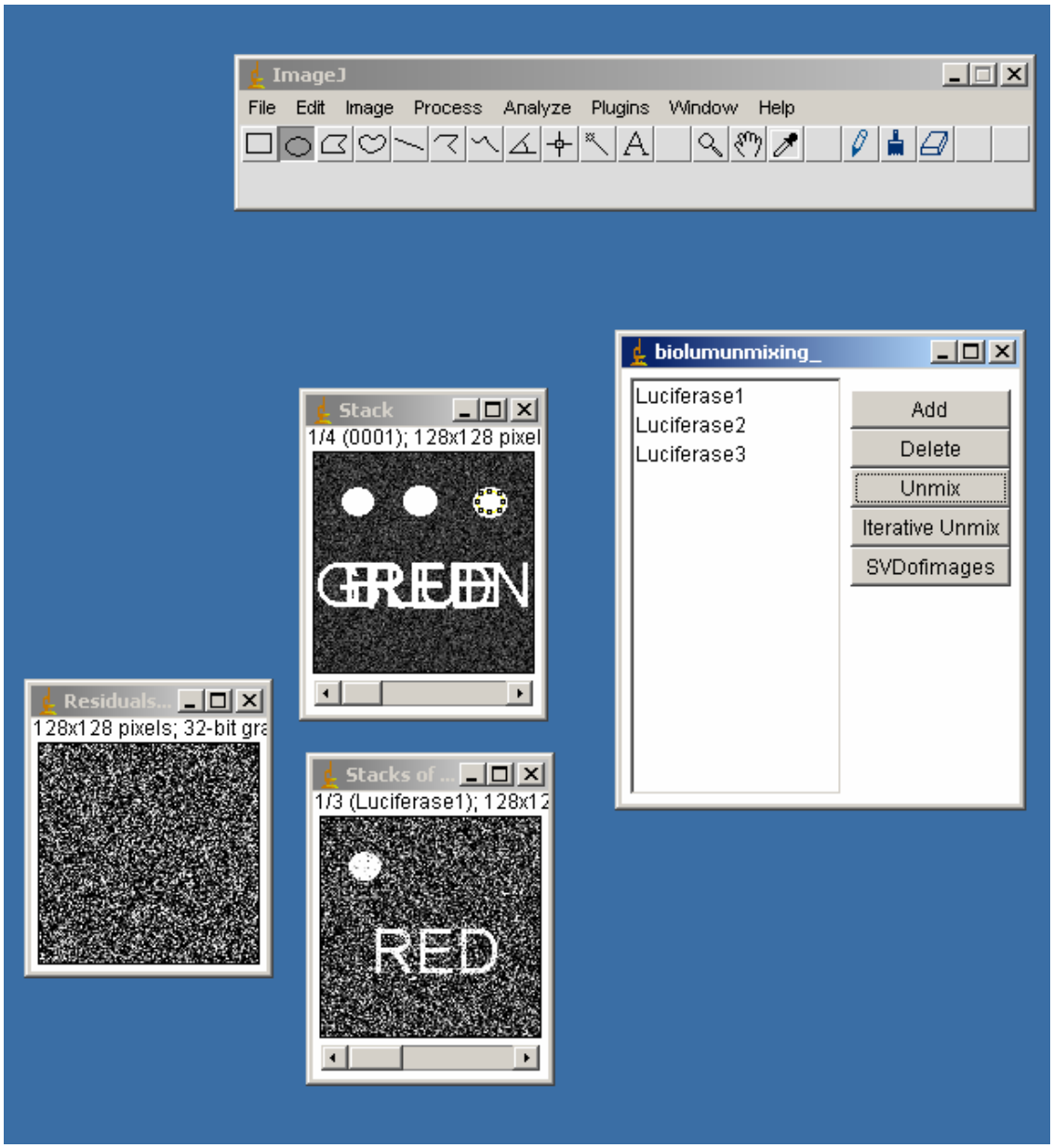


6) Repeat this for each luciferase present in the image.
*NOTE: ! This a critical step in the deconvolution process do not include pixels that are dominated by machine noise in the ROI. Zoom in tight around the region and use the segmented line selection tool to generate a precise ROI. !

7) This will build up a list of luciferases, for example from Luciferase1 to Luciferase 3. You can delete any or all of the luciferases by highlighting the name in the list clicking the delete button.
8) Also you can re-use ROIs that have been saved in the ROI manager by selecting an ROI from the ROI manager list and then clicking Add on the Biolumunmixing window.
9) After all of the luciferase control wells have been selected click Unmix.



10) The program will then return the conditional value of the spectral matrix. This is the sensitivity of the unmixing to error. The higher the number, the more noise will be propagated into the deconvoluted images. I don't recommend quantifying images with conditional values greater than 9. I get good quantification with values between 1 and 4.
11) Biolumunmixing will then calculate and return, an image containing of the residual values and a stack of the spectrally unmixed luciferases. The brightness and contrast will be the same as in the original stack.



12) The stack of unmixed images will be labeled Luciferase1, Luciferase2, etc. The image of Luciferase1 displays the light emitted from the luciferase which was labeled as Luciferase1 when added to the list of luciferases using the Add button.
13) The number of luciferases is limited only by the memory allocated to ImageJ and the spectral resolution offered by your filters.

General Notes on Experimental Design:

1) All of the normal rules of good biochemistry apply. Make sure that you have temperature equilibrated your samples and there is equivalent volume of buffer in your wells.
2) You need to acquire an open image and at least one filtered image (you can acquire more) for each luciferase present in the image.
3) If one reagent strongly absorbs in the visible spectrum make sure and include this reagent in your luciferase control wells.

4) Generally acquire images so that your signal to noise in the open image is about 10:1 or greater.
5) You can also use the control wells to estimate the crosstalk present in the unmixed images.

Citation:

If you use this program to generate a publication please cite the following reference:

1: Gammon ST, Leevy WM, Gross S, Gokel GW, Piwnica-Worms D. Related Articles, Links

Spectral unmixing of multicolored bioluminescence emitted from heterogeneous biological sources.
Anal Chem. 2006 Mar 1;78(5):1520-7.
PMID: 16503603 [PubMed - in process]